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2. Patent application number *(The Patent Office will fill in this part)* — 15 MAY 1998 — 9810423-5

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED
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Patents ADP number *(if you know it)* 3984150002

If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM

4. Title of the invention

IONIZING RADIATION OR DIATHERMY-SWITCHED GENE THERAPY VECTORS AND THEIR USE IN ANTITUMOUR THERAPY

5. Name of your agent *(if you have one)*

H.N. & W.S. Skerrett
Charles House

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

148/9 Great Charles Street
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United Kingdom

Patents ADP number *(if you know it)* 6452002

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Mr. J.N.H. Skerrett
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# IONIZING RADIATION OR DIATHERMY-SWITCHED GENE THERAPY VECTORS AND THEIR USE IN ANTITUMOUR THERAPY

## FIELD OF THE INVENTION

The present invention relates to the field of molecular biology and gene therapy, especially as applied to cancer therapy.

## BACKGROUND

In connection with cancer therapy it has been suggested that cancer cells may be effectively treated by introducing into them sensitizing foreign genes, the expression of which leads to the destruction or elimination of these cells. This could be achieved, for example, via the expression of a cytotoxic protein or cytotoxic RNA species, or via the expression of an immune-response stimulating factor or of substances that can bring about or promote the bio-conversion and activation of a systemically applied inactive chemical agent or prodrug to form an active cytotoxic drug (see for example Elizabeth A. Austin, *et al.*, (1992), "A First Step in the Development of Gene Therapy for Colorectal Carcinoma: Cloning, Sequencing, and Expression of *Escherichia coli* Cytosine Deaminase", *Molecular Pharmacology*, 43, 380-387). The latter approach has been generally favoured in most cases because the active drug so produced is able to kill cells in the vicinity of the sensitised activating cells (the "bystander" effect), thus compensating for any inefficiencies in cellular uptake of the activating gene expression system.

Difficulties with these gene therapy methods, however, include the fact that most current DNA or gene delivery and transfection systems usually propose the use of genetically engineered viruses which, up to now, have not been able to deliver DNA for therapeutic purposes exclusively to tumour cells. Although there have been attempts to circumvent this problem by employing expression controlling regions (promoter and/or enhancer elements) of genes

that are predominantly expressed in tumour cells to direct the expression of the tumour-cell sensitizing mechanism, it has had to be accepted that such genes can also be expressed in normal tissues so that selectivity of expression is not absolute. Methods for the delivery of the genes specifically to target tumour cells by exploiting cell-specific surface antibodies or receptors have also been considered, but these are also expressed in a number of normal cell types and thus selection is again not absolute. In order to be useful and safe for the treatment of cancer, ideally there needs to be a level or levels of selection that will result in expression of the cytotoxic mechanism in the tumour or in the vicinity of the tumour or in the tumour cells exclusively.

Some methods of cancer treatment depend on ionizing radiation which is very commonly used against a variety of cancers. Devices are widely available for directing the radiation from an external source to the tumour in such a way that the dose of radiation to normal tissues is minimised (conformal radiotherapy). However, problems associated with radiotherapy include the fact that tumour cells can often be more resistant to treatment than normal cells, whilst some normal cell types inside the radiation field may be very radiosensitive. In some alternative attempts to deliver the radiation more specifically only to tumour cells, tumour-targeting antibodies or similar molecules have been labelled with various radioisotopes. In this latter case, however, the amounts of radiation that can be administered overall to the tumour within tolerable systemic levels are often too small to be of sufficient therapeutic value and this approach has therefore been generally considered more suitable for tumour imaging than for therapy.

The term "ionizing radiation" as used herein may include not only radiation such as x-rays and $\gamma$-rays, but also high energy sub-atomic particles such as $\alpha$-particles and $\beta$-rays or electron beams.

Another method of cancer treatment uses diathermy. However, despite extensive trials, hitherto this method has not found widespread use because the response curves for human cells are very steep and because of the difficulties in achieving and maintaining precise and homogeneous elevated temperatures in deep-seated tumours.

A general objective of the present invention is to provide improved methods of selectively killing tumour cells using ionizing radiation or diathermy which can avoid or overcome at least some of the problems encountered in methods previously proposed.

## SUMMARY OF THE INVENTION

The present invention is based on a concept of selectively killing tumour cells by using gene therapy methods to provide the tumour cells with a silenced or dormant killing mechanism that can be activated by ionizing radiation or by heat, e.g. from diathermic devices. It is in fact already known that expression of certain genes in human cells can be induced or upregulated by exposure of the cells to heat or ionizing radiation, in the latter case sometimes after very low doses (see for example D.E. Hallahan *et al.* (1995) "Spatial and temporal control of gene therapy using ionizing radiation", *Nature Medicine*, Vol. 1, No. 8, 786-791) and D. E. Hallahan, *et al.*, (1995), "*c-jun* and *Egr-1* Participate in DNA Synthesis and Cell Survival in Response to Ionizing Radiation Exposure", *The Journal of Biological Chemistry*, 270, 30303-30309), but it is believed that this effect has not previously been exploited in the same way as in the present invention.

More specifically, the invention envisages a method of treatment for cancer patients in which there are delivered to tumour cells vectors, preferably self-replicating vectors, containing a dormant or silenced tumour cell sensitizing gene or genes of which transcription and expression can be initiated by a dose of heat or ionizing radiation to generate a product that will bring

about the death or elimination of said tumour cells, e.g. through activation of a cytotoxic prodrug or other cytotoxic agent or tumour cell destruction or elimination mechanism.

Thus, from one aspect the invention provides a vector containing a dormant or silenced tumour cell sensitizing gene (or genes) and a gene expression regulatory system that includes an ionizing radiation or heat responsive element or elements arranged such that ionizing radiation or heat interacting with tumour cells transformed by said vector can trigger and activate said gene expression regulatory system of said transformed tumour cells and thereby subsequently bring about permanent expression of said tumour cell sensitizing gene (or genes) to yield an expression product that can cause said tumour cells to be killed or eliminated, or that can render said tumour cells at least vulnerable to killing and elimination. In at least some cases it may be sufficient for such heat or ionizing radiation to be applied at sub-therapeutic doses and/or, in the case of ionising radiation, at tumour imaging doses.

The term "gene" is used herein to denote a nucleotide sequence, with or without intervening introns, that encodes a functional protein or RNA molecule. It may therefore embrace cDNA sequences. Also, the term "vector" is used herein to denote an agent or vehicle adapted to act as a carrier of nucleic acid fragments or nucleotide sequences inserted therein for the purpose of introducing such fragments or sequences into a bacterial or mammalian cell. As such, the term "vector" as used herein embraces viruses, including phages, and nucleic acid gene portions thereof, as well as bacterial and synthetic plasmids. Where the vector contains an inserted gene or genes and a regulatory system or promoter which facilitates efficient transcription and, where appropriate, translation of said inserted gene(s), it is termed an "expression vector".

The invention may also be defined as consisting of an expression vector for use in transforming human tumour cells in the course of antitumour therapy, characterised in that the said vector contains a tumour cell sensitizing gene (or genes) and also a gene expression control system adapted to be activated by ionizing radiation or diathermic heat in such a way as to bring about the permanent expression in transformed tumour cells of said tumour cell sensitizing gene(s), yielding an expression product that causes said cells to be killed and destroyed, or to be eliminated, or otherwise inactivated, or to be rendered sensitive and vulnerable to destruction.

In general, the term "tumour cell sensitizing gene" is used herein to denote a gene or any DNA sequence or combination thereof which when expressed *in vivo* in a tumour cell generates a product that is effective in bringing about the destruction or elimination of such tumour cell and possibly other tumour cells in the vicinity or in metastases.

Various kinds of tumour cell sensitizing genes may be used to yield an expression product that will give the desired result. In practice, it will often be preferred to use a tumour cell sensitizing gene (or combination of genes) which yields an expression product that is itself a cytotoxic agent or, more preferably, that is an enzyme or other bioactive agent able to bring about the breakdown or conversion of an inactive prodrug into an active cytotoxic form. For example, the *Herpes simplex* virus thymidine kinase gene may be used to produce, when transcribed and translated, the thymidine kinase enzyme which is able to convert the inactive prodrug gancyclovir into a cytotoxic metabolite. An effective amount of such prodrug may be administered systemically, at the same time as transformed tumour cells are subjected to ionizing radiation or heat treatment, or before this treatment, or subsequent to this treatment.

Instead of or as well as providing tumour sensitizing genes or cDNAs that encode prodrug activating enzymes or other toxic agents, additional or

alternative possibilities include (a) providing DNA sequences or cDNAs that encode immune response stimulating factors intended to bring about the elimination of not only the primary tumour cells but also other tumour cells in tumour metastases, (b) providing DNA sequences encoding ribozymes or RNA molecules that will attenuate the expression of vital proteins or RNA molecules, i.e. any such molecules that are essential for cell survival and propagation, and (c), providing any other cell killing or cell removal mechanisms. Hereinafter, these various DNA sequences will be collectively referred to as "genes", and insofar as they may be administered to a mammal for therapeutic purposes they may all be regarded as covered by the term "therapeutic DNA".

Although it is feasible to arrange for expression of the tumour cell sensitizing gene(s) to be directly under the control of an ionizing radiation or heat responsive promoter or enhancer or other expression-regulatory element of the gene expression regulatory system, a potentially serious practical problem results from the fact that such genes presently known which respond to give the highest levels of ionizing radiation-induced or heat-induced expression do so only transiently. This suggests that in order to achieve effective treatment it would be necessary to apply continuous or repetitive exposure to heat or ionizing radiation and also, in the case in which prodrugs are employed, to apply such prodrugs at a particular time for appropriate levels of prodrug activation to occur. Whilst continuous exposure to ionizing radiation at low doses sufficient for activating radiation responsive promoter elements may be achieved using a suitable radioactive isotope-labelled tumour specific antibody or ligand, prima facie it would appear that any antitumour treatment methods based on exploiting the response of radiation responsive or heat responsive promoter elements to ionizing radiation or to heat in a gene therapy scenario would necessarily be of limited value. However, in embodiments of the present invention herein presented the vector is so constructed that expression of the

tumour cell sensitizing gene(s) is only indirectly under the control of a radiation or heat responsive element or elements of the gene expression control or regulatory system, and the radiation or heat responsive element or elements is or are arranged to have the effect, when activated, albeit transiently, of triggering permanent expression of the tumour sensitizing gene(s).

Permanent expression is achieved in accordance with the invention by constructing the vector so that activation of the radiation or heat responsive element(s) of the expression control or regulatory system induces expression of another gene in the vector which encodes an enzyme that acts to modify the vector, for example through a site-specific recombination system, in such a way as to switch on continuous expression of the tumour-sensitizing gene(s).

Thus, in preferred embodiments, the vector has an ionizing radiation or heat responsive promoter element or elements operatively linked to a gene that encodes a recombinase enzyme that is able to bring about the recombination of individual short segments of specific DNA sequences in another region or copy of the vector which in turn brings about a resultant activation and expression of the tumour cell killing or sensitizing gene(s). These above-mentioned specific DNA sequences constitute recombinase target sites and preferably they flank a region which contains transcription termination or stop sequences forming a so-called "Stop cassette" (available for example in Gibco™ plasmid pBS302) or other intervening sequence that prevents expression of the downstream sequences (Gibco and Gibco BRL are trade marks of Gibco Europe Limited and/or Life Technologies, Inc.). This is located upstream of the tumour sensitizing gene but downstream of a separate promoter sequence whereby expression of this tumour sensitizing gene is normally prevented. The vector can be constructed, however, so that the recombination of the DNA sequences of the recombinase target sites results in the elimination or deletion of this Stop

cassette which is excised from the vector so that transcription and expression of the tumour killing or sensitizing gene(s) is then no longer prevented.

It will be understood that the heat or radiation responsive promoter element or elements generally will be such that exposure to heat or ionizing radiation elicits a response that brings about a substantial, or at least effective, increase in activity and hence transcription and translation of downstream sequences. Preferably the expression control or regulatory system is such that this response can be brought about by very low, non-therapeutic, sublethal doses of heat or ionizing radiation. Following activation by such treatment, this heat or radiation-responsive promoter or element(s) will then cause expression of the gene encoding the recombinase enzyme that brings about the sequence-specific recombination of the recombinase target sites which are located in another region of the vector and which flank the region referred to as the Stop cassette.

Suitable recombinase genes that may be used in this arrangement include the *Escherichia coli* P1 Bacteriophage *cre* and the *Saccharomyces cerevisiae flp* recombinase genes. Other genes with similar characteristics could also be used.

It should be pointed out that as the gene expression regulatory or control system in eukaryotic cells may comprise a relatively complex promoter consisting of a number of different, non-contiguous, separate parts, and may also include a more remotely located enhancer sequence associated with the promoter, the term promoter element or elements used herein is to be construed broadly as denoting any appropriate part of the expression regulatory or control system.

The *cre* gene, which expresses the Cre recombinase protein of bacteriophage P1, is used in conjunction with *lox*P target or recombination sites. The Cre-loxP site-specific recombination system is a well known

recombination system (see for example Martina Anton, *et al.*, (1995), "Site-Specific Recombination Mediated by an Adenovirus Vector Expressing the Cre Recombinase Protein: a Molecular Switch for Control of Gene Expression", *Journal of Virology*, 69, 4600-4606, and Minmin Qin, *et al.*, (1995), "Site-specific cleavage of chromosomes in vitro through Cre-Lox recombination", *Nucleic Acids Research*, 23, 1923-1927), and the elements thereof, including the *cre* gene, *lox*P sites and Stop cassette assembled in plasmid vectors, are commercially available, e.g. as a Gibco BRL™™ product from Life Technologies, Inc. (U.S.A.). The Flp site specific recombinase system in conjunction with FRT target sites that provide substrates for the Flp recombinase protein (see for example Dymecki (1996) "Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice", *Proc. Natl. Acad. Sci. U.S.A.*, 93, 6191-6196) may be applied in a similar way to excise a blocking Stop cassette so as to "switch on" expression of the tumour sensitizing gene after activation of the *flp* recombinase gene which is arranged to be under the control of a radiation or heat responsive promoter in the vector.

Examples of promoters or expression control elements that can be activated by low doses of ionizing radiation include the enhancers and/or promoters or expression control radiation responsive elements of the *egr-1* gene, TNFα gene, the *Nfkβ* gene, the *c-fos* gene, the *jun-b* gene, the *c-jun* and *the c-myc* gene. This list, however, is not exhaustive. A typical example of a heat responsive promoter that may be used is the *hsp-90* gene.

As indicated above, transcription and translation of the tumour sensitizing gene(s) in the vector generally will be under the control of a separate promoter. This is preferably a promoter that operates very effectively in human cells and most preferably one that will operate specifically in the type of cells that make up the tumour to be treated, by virtue for example of being

tissue or cell-type specific or being associated with a tissue specific enhancer region, or even more preferably one that operates only in tumour cells. This second promoter for activating the tumour sensitizing gene(s) should be located in the vector in such a position that it does not bring about the expression of any gene at the protein level until a recombination of the DNA sequences of the recombinase target sites occurs. Thus, this second promoter may be located upstream of the Stop cassette which, as pointed out above, is itself located upstream of the tumour-sensitizing gene(s) so as to block expression of the latter. Alternatively, this second promoter may be located within a region excised by the action of the recombinase enzyme such that upon recombination this second promoter is reorientated so as to be able to promote continuous transcription and translation of said tumour-sensitizing gene(s) co-excised with the second promoter.

Examples of mammalian cell promoters that will generally be suitable include the human cytomegalovirus (CMV) gene promoter and the chicken B-actin gene promoter, whilst one example of a tissue specific promoter (in this case appropriate for treating prostatic tumours) is the prostate specific antigen promoter and its associated enhancer region (see for example E. R. Schuur, *et al.*, (1996), "Prostate-specific Antigen Expression Is Regulated by an Upstream Enhancer", *The Journal of Biological Chemistry*, 271, 7043-7051). Examples of tumour-specific promoters, in these cases acting in tumour cells that have lost p53 function, include the HSP-70 promoter (Tsutsumi-Ishii *et al.*, (1995) *Cell growth and differentiation*, 6, 1-8) and the MDR-1 promoter (Zastawy, R.L. (1993) *Oncogene* 8, 1529-1535). For tumour cells that have lost RB-1 function the E2F-1 promoter would be appropriate (M.J. Parr *et al.*, (1997) (see "Tumour-selective transgene expression in vivo mediated by an E2F-responsive adenoviral vector", *Nature Medicine*, 3, 1145-1149).

The vector will also usually be constructed in a known manner to provide, as necessary for optimal effect, a polyadenylation signal encoding region at the downstream ends of the recombinase and tumour sensitizing gene(s) and mammalian cell origins of replication. For amplification in bacteria, the vector will also contain a bacterial origin of replication and an antibiotic resistance gene, as understood by those versed in the art.

As already mentioned, preferred tumour-sensitizing gene(s) include genes that encode prodrug activating enzymes, but other examples are genes that encode cytotoxic proteins or toxins, immune response stimulating factors, ribozymes or antisense RNA molecules. Apart from the *Herpes simplex* virus (HSV) thymidine kinase (*tk*) gene previously mentioned, further examples of tumour sensitizing prodrug activating genes that can be suitable include the *E.coli* nitroreductase (*nr*) gene (see S. M. Bailey *et al.*, (1996) "Investigation of alternative prodrugs for use with *E. coli* nitroreductase in 'suicide gene' approaches to cancer therapy" *Gene Therapy*, 12, 1143/1150), the cytosine deaminase gene, and the mammalian cytochrome p450 2E1 or 2DVI genes. Other genes that encode proteins involved in different cell killing mechanisms can also be useful.

The vector for delivery of the therapeutic DNA to patients can be a retroviral, lentiviral, adenoviral, adenovirus-associated viral, or an Epstein-Barr viral based vector or any viral or bacterial vector delivery system that might be used for gene therapy in humans. Alternatively, it can be a non-viral vector that would be made up for administration in a suitable formulation such as, for example, a complex with cationic liposomes or with a tumour-targeting antibody or ligand, or that would be incorporated into some other non-viral DNA delivery system for delivery to human tissues, especially to tumour cells. In general, the vector will have the ability to invade tumour cells and to express the encoded tumour sensitizing genes(s) therein following exposure of

transformed cells to diathermy or low doses of ionizing radiation. Delivery of the vector will usually be carried out according to generally accepted gene therapy procedures or methods as described, for example, by Friedman in *Therapy for Genetic Disease*, T. Friedman, ed., Oxford University Press (1991) and reviewed by I.M.Verma and N. Somia (1997) in "Gene therapy-promises, problems and prospects" *Nature* 389, 239-242. For example, after constructing the viral or plasmid vector containing the tumour cell sensitizing gene(s) and expression control element(s) described, the vector may be incorporated in a pharmaceutical composition, possibly in combination with a pharmaceutically acceptable excipient or carrier vehicle (including for example a transmembrane carrier such as Penetratin™), which may then be injected into the patient, either locally at the site of the tumour or systemically. Such pharmaceutical compositions or formulations represent a further aspect of the invention.

As indicated above, gene transfer methods known in the art which may be useful in the practical application of the present invention may include both viral and non-viral transfer methods. Viruses that have been used as gene transfer vectors include for example papovaviruses, vaccinia viruses, herpesviruses, as well as adenoviruses, lentiviruses, adeno-associated viruses and retroviruses of avian, murine and human origin. Many human gene therapy protocols have been based on disabled murine retroviruses.

Non-viral DNA transfer methods known in the art include mechanical techniques such as micro-injection, membrane fusion-mediated transfer via liposomes (as already mentioned), and direct DNA uptake and receptor-mediated DNA transfer. Also, viral-mediated DNA transfer can be combined with direct *in vivo* DNA transfer using liposome delivery which may allow one to direct the viral vectors to the tumour cells concerned rather than into the surrounding normal cells. Alternatively, one may inject into tumours a self-replicating retroviral vector producer cell line so that there could then be a

continuous source of DNA vector particles, similar to a technique already approved for use in humans with inoperable brain tumours. Although the vectors may be taken up directly by cells, actively or by diffusion, liposome mediated transfer may be best achieved in some cases by use of a transfection agent such as a cationic lipid, e.g. the compound N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium methylsulfate, commonly known as DOTAP, marketed by Boehringer Mannheim. However, many other suitable methods will be known to persons familiar with gene therapy techniques. An important aspect of the invention is that the delivery system does not need to have absolute specificity for tumour cells since the tumour cell killing mechanism will only be activated in the area affected by radiation or diathermy, and may in some embodiments only operate in cancer cells by virtue of tumour-specific promoters driving the expression of the tumour-sensitising gene(s).

For clinical use, the vectors may be mixed with a selected transfection agent to provide a pharmaceutical preparation which may be administered by any suitable means, for example parenterally, orally or perhaps topically. In at least some cases such pharmaceutical preparations will be in the form of a sterile liquid formulation, presented possibly in unit dosage form in sealed ampoules ready for use, and as already mentioned delivery or administration may be effected by injection, e.g. directly into tumour tissue or intravenously. In practice, not only the method of administration but also the particular protocol employed may be important; however, the precise details of the treatment and appropriate dosages will generally be determined by carrying out straightforward trials and by the general experience of the medical practitioners in charge of the treatment.

The invention also extends to methods for treating tumour cells in a host which, at least in one embodiment, comprises:

(a) administering to the host a composition comprising a vector containing a tumour cell sensitizing gene or genes and having an ionizing radiation or heat responsive gene expression control system operatively linked to a recombinase gene, together with recombinase target sites flanking a region of which removal permits continuous expression of said tumour cell sensitizing gene(s) as specified above, the amount of said composition administered being effective for transforming said tumour cells;

(b) causing said tumour cells to be subjected to a dose of diathermy or of ionizing radiation effective to activate the recombinase gene expression control system of said vector, thereby to bring about, via recombinase-mediated site specific recombination within the vector, expression of the or each tumour cell killing or sensitizing gene(s) component of the vector;

and, in the case of prodrug activating genes,

(c) administering to the host an effective amount of a composition comprising a prodrug convertible into an active form by the expression product of said tumour cell sensitizing gene or genes.

Usually, in most embodiments, the dose of diathermy or of ionizing radiation will be derived from a directed external source or from radioisotopically labelled tumour-seeking or cell or tissue-type seeking agent. Examples of suitable radioisotopically labelled tumour-seeking agents for use in the above method include materials such as metaiodobenzylguanidine or suitably radioisotopically labelled tumour cell specific antibodies or ligands.

The prodrug referred to above may comprise any of the following compounds, administered either singly or in appropriate combinations: gancyclovir, CB1954, fluorouracil, dacarbazine, or cyclophosphamide, subject

to the prodrug activating gene(s) being, respectively, a gene or genes or cDNAs encoding HSV thymidine kinase, nitroreductase, cytosine deaminase, cytochrome p450 2El or cytochrome p450 2DVl.

To summarise, in general in preferred embodiments:

(1) where an ionizing radiation responsive expression control system is used, the radiation responsive element or elements is selected from the radiation responsive enhancer or promoter of the *egr*-1 gene, the *TNFα* gene, the *Nfkβ* gene, the *c-fos* gene, the *jun-b* gene, the *c-jun* the *c-myc* gene and their functional equivalents, and either a single such radiation responsive element may be used or a plurality of tandemly arranged radiation-responsive DNA sequence elements (all the same or different) may be used in an array thereof;

(2) where a heat-responsive promoter or expression control element is used, this is the promoter of the *hsp-90* gene or its functional equivalent;

(3) the recombinase gene contained in the vector will be the *E.coli* bacteriophage P1 *cre* gene or the *Saccharomyces cerevisiae FLP* gene, or a functional equivalent of one or other of these genes;

(4) the DNA sequence of the vector will include polyadenylation signal encoding regions from the virus SV40 or equivalent inserted at appropriate locations;

(5) the vector may contain a mammalian cell promoter which is that of the cytomegalovirus (CMV) or that of the chicken B-actin gene, or a functional equivalent of these;

(6) the vector may contain a tissue specific promoter (e.g. the prostate specific antigen promoter and its associated enhancer region, or an

equivalent of this) that is specific for the particular tumour type to be treated;

(7) the vector may contain a tumour specific promoter such as the HSP-70 promoter, the MDR-1 promoter, the E2F-1 promoter or telomerase-related promoters (or their equivalents), according to the p53 or RB-1 or telomerase status of the tumour;

(8) the DNA vector will contain recombinase target sites provided by the *lox*P sites (or equivalents) that are substrates for the Cre recombinase enzyme, or recombinase target sites provided by FRT sites (or their equivalents) that are substrates for the Flp recombinase enzyme, or equivalents;

(9) the vector will contain a Stop cassette provided by a sequence that prevents expression of any protein coding sequence located downstream thereof unless this region is excised by the action of the recombinase, or by another mechanism that results in a change in orientation or loss of this Stop sequence;

(10) the tumour sensitizing gene is selected from the *E.coli* nitroreductase gene, cytosine deaminase (CD) gene, *Herpes simplex* virus thymidine kinase (HSV-*tk*), mammalian cytochrome p450 2E1 or 2DVI gene, and their functional equivalents;

(11) The vector will contain a bacterial origin of replication;

(12) The vector will contain at least one mammalian cell origin of replication;

(13) The vector will contain a bacterial antibiotic resistance gene;

(14) The vector will contain intron sequences;

(15) The vector may contain a mammalian cell antibiotic resistance gene.

By way of example of the manner in which the invention may be carried out illustrative embodiments and background work in developing the invention will now be described in more detail with reference to the accompanying drawings. The particular embodiments and examples illustrated and described, however, should not be construed in any way as a limitation on the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Schematic diagrams (not to scale) of vectors in accordance with the invention and containing the above-mentioned elements are shown in the accompanying drawings. In these drawings:

FIGURE 1 represents one embodiment of a gene therapy plasmid vector, herein labelled "pComplete1", containing an ionizing radiation responsive recombinase expression control system;

FIGURE 2 shows some of the components making up pComplete2, a modified version of the vector in Figure 1;

FIGURE 3 shows some of the components making up pComplete3, another version of the vector illustrated in Figure 1;

FIGURE 4 is a diagram showing different stages in the construction of the pComplete1 vector;

FIGURE 5 is a schematic diagram demonstrating how pComplete1 responds to ionising radiation, undergoes recombination, and permanently expresses the tumour sensitising gene;

FIGURE 6 is a diagram showing stages in the construction of vectors pEGRL(b)-*cre* and pStop-*gfp* used in testing the principle of the invention;

FIGURE 7 is a diagram showing FACS analysis of MCF-7 cells following transfection with pStop-*gfp* only or pStop-*gfp* together with

pEGRL(b)-*cre* and exposure to 5Gy or 10Gy (in two 5Gy doses) of ionising radiation. Controls were not irradiated (0Gy); and

FIGURE 8 is a diagram showing the results of a further test in which MCF-7 cells were transfected with pEGRL(b)-*cre* and selected using the antibiotic, G418, giving rise to the cell clone pCE which was, in turn, transfected with pStop-*tk* and then subjected to radiation, in the absence of and in the presence of the prodrug ganciclovir.

## DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS

Referring to the drawings, in the particular example of the plasmid vector pCompletel depicted in Figure 1 the ionizing radiation responsive recombinase expression control system is provided by a promoter sequence comprising a synthetic tandem array of radiation-responsive elements corresponding to radiation-responsive elements found in the promoter of the *egr*-1 gene, and is located upstream of the cytomegalovirus immediate-early (CMV I.E.) promoter. A recombinase gene protein coding sequence or open reading frame (ORF), which in this example is that of the P1 bacteriophage *cre* gene (labelled *Cre* ORF), is located downstream of an intron sequence, the latter serving to enhance the translation of the downstream ORF. There is also in this plasmid vector a mammalian cell enhancer/promoter which is that of the cytomegalovirus gene (labelled CMV I.E. Enhancer/promoter), *lox*P recombinase target sites that provide substrates for the Cre recombinase enzyme, and a tumour sensitizing gene which in this example is the Herpes simplex virus thymidine kinase (*tk*) gene (labelled *tk* ORF). As indicated, the *lox*P sites flank a Stop cassette. Downstream of each ORF is located a polyadenylation signal-encoding sequence which in this case is derived from the Simian virus 40 (SV40) early gene and is labelled SV40 polyA site in the diagram of Figure 1.

The term enhancer/promoter is used herein to denote combined or fused enhancer and promoter nucleotide sequences.

Following recombination, the above-mentioned mammalian cell enhancer/promoter drives expression of the *tk* gene. Possible alternatives include the promoter of the chicken B-actin gene, a tissue specific promoter such as for example the prostate specific antigen promoter and its associated enhancer region, or a tumour specific promoter such as for example the HSP-70 promoter, the MDR-1 promoter, the E2F-1 promoter or telomerase-related promoters.

As pointed out, other possible tumour sensitizing genes that may be used include the *E.coli* nitroreductase or cytosine deaminase genes, and the mammalian cytochrome p450 2El or 2DVI cDNAs. Possible alternatives for the above-mentioned radiation-responsive promoter for the recombinase gene include promoters comprising different numbers of radiation-responsive elements, which may be the same or different, arranged in tandem, the entire enhancer/promoter DNA sequence, or only the enhancer DNA sequence of the *egr*-1 gene. Other alternatives include the sequences of the *TNFα* gene, the *Nfkβ* gene, the *c-fos* gene, the *jun-b* gene, the *c-jun* and the *c-myc* gene or their functional equivalents. Where a heat-responsive promoter or expression control element is used, as previously mentioned this can be the promoter of the *hsp-90* gene or its functional equivalent.

In Figure 1 the region labelled "Neo gene" indicates the amino-glycoside phosphotransferase gene ORF complete with a mammalian cell promoter and SV40 polyadenylation site, which is incorporated for selective growth of transfected mammalian cells *in vitro* in the selective antibiotic G418. This allows the vector to be used as a model to demonstrate aspects of the invention *in vitro* in cultured human cells since it allows permanent cell lines to be established. Such cell lines, e.g. the pCE cell line (see later), can be used not

only to demonstrate the working of the invention *in vitro* but they can also be used to produce tumour xenografts in immune-deficient mice for demonstrating the working of the invention *in vivo*. Also as shown in Figure 1, the plasmid contains an antibiotic resistance gene expression cassette such as that conferring resistance to ampicillin in *E.coli*, (labelled Amp in Figure 1), a bacterial origin of replication for growth of the plasmid in *E.coli* (labelled *E.coli* ori in Figure 1), and a mammalian cell origin of replication for replication of the plasmid in mammalian cells (not shown). The latter would be ideally located outside the region excised by the recombinase so that additional copies of the inactive plasmid might be produced by DNA replication prior to recombination, and following recombination additional copies of the activated plasmid, now expressing the tumour killing or sensitising gene(s), might also be produced by DNA replication, resulting in increased expression of the tumour killing or sensitisation gene(s).

The vector illustrated in Figure 2 (pComplete2) contains the same elements as are shown in Figure 1 but the region between the *lox*P sites has been modified to contain another copy of the Cre ORF and of the SV40 poly A site, the direction of transcription of these sequences being opposite to that of the *tk* gene. Another copy of the CMV I.E. enhancer/promoter is located adjacent to the Stop cassette and there is another intron located transcriptionally upstream of this. These elements are positioned such that excision and recombination of the region between the *lox*P sites by Cre (supplied by a radiation-upregulated Cre expression cassette located elsewhere in the plasmid as shown in Figure 2) results in the production of two circular plasmid molecules, the *tk* gene being expressed from one, while the *cre* gene in the other is now able to be expressed as a result of the repositioning of the CMV I.E. enhancer/promoter upstream of the Cre ORF. This should ensure that any copies of the switchable plasmid which are present in the cells but which have

not become activated by the initial removal of the Stop cassette would then be activated, without the requirement for additional doses of radiation or diathermy. Again, the Cre-expressing circularised fragment can be designed to contain a mammalian cell origin of replication so that additional copies of the activated plasmid might be produced by DNA replication, resulting in increased expression of Cre.

The vector in Figure 3 (pComplete3) is designed so that that excision of the Stop cassette region between the *lox*P recombination sites generates a DNA fragment in which two different tumour sensitising (or killing) genes flanking an internal ribosome entry site (IRES) come under the influence of a mammalian cell enhancer/promoter or tumour-specific enhancer/promoter also contained within that fragment, giving rise to continuous expression of both these two genes. The example shown in Figure 3 is of the *tk* gene and the E.coli nitroreductase gene (labelled Nitroreductase-ORF) that would be expressed by virtue of the CMV I.E. enhancer/promoter, following Cre-mediated recombination.

It will be appreciated that many variations of the above-described vectors can be produced. For example, in pComplete2 the positions of the Cre ORF and *tk* ORF flanking one of the *lox*P sites can be reversed so that, following the action of Cre, the shorter region between the *lox*P sites will express the tumour sensitising gene whilst the residual vector will continuously express Cre. Furthermore, additional IRES will allow the expression of more than one protein or other tumour cell killing molecule from both of the vector fragments following recombination and both fragments might contain a variety or mixture of the Cre ORF and many other tumour sensitising genes, and/or tissue or tumour-specific promoters or enhancer/promoters.

Vector Construction

By way of example, the manner of construction of the pComplete1 vector in accordance with the invention will now be described in somewhat greater detail.

The elements of the vector are assembled in standard ways well-known in the art of recombinant DNA technology from components available commercially or that can be made synthetically or derived by PCR-amplification of human DNA, and appropriate restriction endonuclease sites are introduced which can be used to produce fragments with compatible termini. As hereinafter more fully described, these may then be ligated together as required.

The components of the vector(s) in this specific example are conveniently obtained as summarised below:

(a) The or each tandem array of radiation responsive promoter elements corresponding to the radiation responsive promoter element of the *egr-1* gene is conveniently synthesised initially as a short single-stranded oligonucleotide as described by Weichselbaum *et al.* (1994)) "Gene therapy targetted by radiation preferentially radiosensitizes tumour cells", *Cancer Research,* 54, 4266-4269, the content of this paper being incorporated herein by reference. The terminal regions of this single-stranded oligonucleotide can be designed so that, following annealing to a suitable complementary oligonucleotide, the resulting double-stranded molecule can be ligated into the plasmid vector pCI-*neo* (commercially available from Promega) following its digestion with appropriate restriction endonuclease/s, in this case *Bgl*II and *Sgf*I.

(b) The Cre recombinase protein coding sequence is readily obtained as a *Xho*I/*Mlu*I fragment from the commercially available plasmid pBS185 (Gibco BRL™ - Life Technologies, Inc.).

(c) The cytomegalovirus (CMV) immediate-early (I.E.) enhancer/promoter, SV40 polyadenylation signal, the *E.coli* and mammalian cell origins of replication, ampicillin resistance gene and aminoglycoside resistance cassette (comprising the CMV I.E. enhancer/promoter, aminoglycoside phosphotransferase ("neo") protein coding sequence and SV40 polyadenylation signal) are also conveniently obtained from the plasmid vector pCI-neo commercially available from Promega.

(d) The cytomegalovirus (CMV) immediate-early (I.E.) enhancer/promoter, and a suitable multiple cloning site (MCS) is obtainable as a *Bam*Hi/*Bgl*II fragment from plasmid pCI, also commercially available from Promega.

(e) The *lox*P-flanked Stop cassette can be obtained as an *Eco*Ri/*Spe*I fragment from Gibco™ plasmid pBS302.

(f) The *Herpes Simplex* virus *tk* gene has the nucleotide sequence originally published by Wagner, M.J. *et al.*, (1981) "Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type-1", *Proc. Natl. Acad. Sci. USA*, 78, 1441-1445 and for the purpose of the present invention it is conveniently cloned by standard methods into plasmid pBR322 (Gibco) to produce a plasmid vector here termed pBR-*tk* which provides a carrier and reservoir of the gene.

More specifically, the production of the vector pComplete1 shown in Figure 1 from synthetic oligonucleotides, plasmids pCI-neo and pCI (Promega), plasmids pBS302 and pBS185 (Gibco) and the above-mentioned pBR-*tk*

plasmid can be carried out in a series of stages substantially as described below with reference to the scheme illustrated in the diagram of FIGURE 4, and with reference to the oligonucleotide sequences shown in TABLE 1 at the end of the present description.

In the diagram of FIGURE 4, the double-framed boxes indicate starting materials, and the various stages may be carried out substantially as follows:

(1) The CMV I.E. enhancerfragment is removed from plasmid pCI-neo following digestion with *Bgl*II and *Sgf*I and is replaced with a double stranded oligonucleotide labelled "RR-elements(a)". This contains a tandem array of 6 radiation-responsive elements and is produced by annealing synthetic oligonucleotides EGRE1 and EGRE2 (see Table 1) to generate the plasmid, labelled pEGRL(a)-neo.

(2) The *cre* recombinase gene protein coding region, obtained as a *Xho*I/*Mlu*I fragment of pBS185, is inserted into *Xho*I/*Mlu*I-digested pEGRL(a)-neo to generate pEGRL(a)-*cre* .

(3) The NotI site in the pEGRL(a)-*cre* Multiple Cloning Site (MCS) is deleted by *Not*I digestion, then the resultant single-stranded termini filled-in using the Klenow enzyme, followed by self-ligation of the plasmid.

(4) The CMV I.E. promoter/enhancer along with its associated multiple cloning site is obtained as a *Bam*HI/*Bgl*II digest fragment from plasmid pCI, and is ligated into the NotI-deleted pEGRL(a)-*cre*, following partial digestion of the latter with *Bam*HI, thereby to produce pEGRL*cre*MCS2.

(5) The Stop cassette, obtained from the Gibco™ plasmid pBS302 by digestion with *Eco*RI and *Spe*l, is ligated into *Eco*RI/*Xba*I digested pCl-*neo* plasmid to generate pStop.

(6) The *tk* gene protein coding region is obtained as a PCR amplification product of pBR-*tk* using the primers Cla*tk* (SEQ ID No: 9) and *tk*Not (SEQ ID No: 10) shown in TABLE 2 at the end of the present description. Following *Cla*I and *Not*I digestion the PCR product is ligated into *AccI/Not*I-digested pStop to generate pStop-*tk*.

(7) The *Not*I/*Eco*RI fragment of pStop-*tk* containing the Stop cassette and the *tk* ORF is ligated into *Eco*RI/*Not*I-digested pEGRLcreMCS2 to generate pComplete1.

Radiation Switched Operation

Figure 5 illustrates in relation to pComplete 1 the molecular switching process in which Cre recombinase transcription and translation is induced following exposure to ionising radiation. The Cre enzyme expressed acts upon pComplete1 to excise the Stop cassette by recombination at the *lox*P sites. This results in the formation of a small circular DNA fragment containing the Stop cassette and a *lox*P site and of a recircularised plasmid (pComplete1: activated form) that transcribes and translates the thymidine kinase DNA sequence. The expression of this enzyme can then in turn cause activation of the prodrug, gancyclovir (provided by systemic injection) which will kill host cells and also cells in contact with the host cells (via the bystander effect).

Modified Vectors

Alternative vectors containing different promoter and/or enhancer elements can be constructed by analogous methods. Thus, as indicated earlier, the vectors may be constructed so as to contain the entire radiation responsive *egr-1* enhancer/promoter. This comprises nucleotides -676 to +10 according to

Sakamoto *et al.* ("5' Upstream sequence and genomic structure of the human primary response gene *egr*-1/TIS8" (1991) *Oncogene* 6, 867-871) and can be isolated from human DNA by PCR amplification using the primers EGRE6 (SEQ ID No: 7) and EGRE5 (SEQ. ID NO: 6) shown in TABLE 2. The enhancer region alone (nucleotides -676 to -178) can be isolated from human DNA by PCR amplification using the primers EGRE5 (SEQ. ID NO: 6) and EGRE7 (SEQ. ID NO: 8) shown in TABLE 2. These primers, which are designed to introduce appropriate restriction endonuclease or other suitable sites for cloning, are conveniently synthesised using commercially available oligonucleotide synthesising apparatus. The human DNA required for carrying out the PCR amplification of these radiation responsive sequences is conveniently isolated from HeLa cells by a conventional method, as described for example by Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989), "Molecular Cloning - A Laboratory Manual", *Cold Spring Harbor Laboratory Press, U.S.A.*).

Alternative recombinase genes and target sites as well as other tumour sensitizing mechanisms can also be used. As previously mentioned, one example of the latter is the *E.coli* nitroreductase gene in respect of which the protein coding sequence (nucleotides 166 to 831) can be isolated by PCR amplification of *E.coli* strain B DNA using oligonucleotide primers designed to introduce suitable restriction endonuclease sites for cloning purposes.

In modifications in which the components of the vectors are arranged in different ways in order to achieve the required effect of radiation (or diathermic) switching, as has been described in relation to the vector illustrated in the diagram of Figure 2 the region between the recombinase (*lox*P) target sites can contain a second copy of the recombinase gene and another mammalian cell promoter on opposite sides of the Stop cassette, the arrangement being such that the recombination and re-orientation brought about

by the action of the recombinase initially expressed will bring this second copy of the recombinase gene under the influence of this other promoter, thereby resulting in the continuous expression of this second copy of the recombinase coding sequence. As has already been pointed out, continued expression of the recombinase enzyme after triggering by a single dose of radiation will ensure that further radiation or heat treatment will not be required if there were to be inadequate or inefficient recombination of the vectors in the tumour cells due to low levels of heat or radiation-induced expression of the recombinase after the initiating dose of radiation or heat. In this way the dose of heat or radiation required to trigger the removal of the Stop cassette and the activation of the silenced expression cassette or tumour sensitizing gene(s) in every copy of the vector taken up by the cells can be kept minimal. This can be particularly important if the system of vector delivery results in the uptake of more than one copy of the vector into the host cells or if the vector undergoes replication before recombinase-mediated recombination.

Additionally, multiple killing or sensitisation factors can be expressed in a similar way by means of internal ribosome entry sites (IRES) that allow the expression of more than one protein coding sequence under the influence of a single promoter region, as has been described in connection with the vector of Figure 3. This can allow, for example, the simultaneous expression of different prodrug activating proteins and immune response stimulating factors, or different combinations thereof, or different combinations of prodrug activating proteins and other proteins, including Cre, or other molecules that kill cancer cells. It is also possible to express fusion proteins having more than one functional prodrug activating activity.

The vectors may be designed to be either episomal, requiring for example an Epstein-Barr virus nuclear antigen-1 gene element and origin of replication, or a SV40 origin of replication. Alternatively, they may be

designed to integrate into host cell DNA as would be the case for retroviral or adenoviral vectors. The excised region between the recombinase target sites or the region outside the recombinase target sites encompassing the tumour killing or sensitising gene(s) may also be designed to be episomal and to have its own mammalian cell origin of replication. It is recognised in some cases that the capacity of certain viral genomes to harbour DNA is size-limited and therefore some components of the vectors herein illustrated may need to be eliminated in order that the essential genetic information can be accommodated.

Testing

The operation of the system can be tested in cultured human tumour cells and xenografts established therefrom using vectors of the general kind herein described.

*(a)* *Demonstration of radiation-mediated switching in human tumour cells*

In order first to prove the switching principle of the invention, a series of experiments was carried out to test the operation of the various components individually and then in combination. For convenience and for the purposes of accurate quantitaton of any effect observed, instead of a prodrug activating gene, the gene encoding the Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* was used as a "reporter" gene. The ORF of this gene was cloned into the vector pStop (see Figure 6) downstream of the Stop cassette to generate pStop-*gfp*. This plasmid, which should not be able to express GFP unless it undergoes recombination, was transfected into MCF-7 cells in order to provide a control.

In constructing pStop-*gfp*, the Green Fluorescent Protein (GFP) open-reading frame was excised from plasmid pEGFP-1 (obtained from Clontech) with *Cla*I and *Not*I and was cloned into the *Acc*I/*Not*I sites of pStop. The radiation-responsive synthetic *Egr-1* enhancer formed by annealing EGRE3 and

EGRE4 (see Table 1) containing four repeats of the previously defined decanucleotide radiation-responsive expression control elements (see Datta *et al.*, (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 101149-101153) was cloned into the pCI-*neo* vector to generate pEGRL(b)-*neo*. The *cre* gene was obtained from pBS185 as an *Xho*I/*Mlu*I fragment and was ligated into *Xho*I/*Mlu*I-digested pEGRL(b)-*neo* to generate pEGRL(b)-*cre*. This, together with the plasmid pStop-*gfp*, was transfected into MCF-7 cell lines. pStop-*gfp*-transfected and pStop-*gfp* plus pEGRL(b)-*cre*-transfected cells were irradiated (5Gy and 10Gy in two 5Gy doses), and the number of fluorescent cells within a constant aliquot of the transfected population was measured by FACS, as hereinafter described. As can be seen in Figure 7, transfection with the pStop-*gfp* vector alone resulted in very low numbers of fluorescent cells, although there was a slight increase in this number following irradiation. Transfection with pStop-*gfp* plus pEGRL(b)-*cre* resulted in a larger number of fluorescent cells than with pStop-*gfp*-transfected irradiated cells, but this number was further increased to a much greater extent following irradiation. It can thus be concluded that irradiation activated the radiation-responsive promoter in pEGRL(b)-*cre* which resulted in the expression of Cre recombinase which in turn recombined the pStop-*gfp* vector, causing expression of GFP. This therefore demonstrated the operation of the general "switch" principle described in connection with this invention.

*(b)* *Demonstration of radiation-mediated killing in human tumour cells*

To test this aspect, MCF-7 cells were transfected as described above with the plasmid pEGRL(b)-*cre* and a permanent cell line, pCE, was established by G418 selection. This was transfected with the plasmid pStop-*tk* described earlier and the cells were plated in 96-well microtitre plates in either complete tissue culture medium (RPMI + 10% foetal calf serum) or the same containing 50 mM ganciclovir (GCV). Eight hours after plating the cells were

irradiated as an attached monolayer ($^{60}$Co gamma rays, approx 1Gy/min) at 37°C. MTT assays were performed 24 hours later and radiation survival data were presented as a mean (± SD) of 48 replicates per dose point.

As shown in Figure 8 there was some degree of cell killing with increasing doses of radiation in the absence of GCV. However, in the presence of GCV, there was substantially more killing at doses of 2Gy or more. This is consistent with radiation-mediated upregulation of the *cre* recombinase gene in the pCE plasmid and subsequent recombination of the *lox*P sites, resulting in the removal of the stop cassette, leading to the synthesis of thymidine kinase and subsequent action of the latter on GCV to convert it to a cytotoxic metabolite. The absence of an effect at the low dose of 1Gy is also consistent with this being below a threshold dose for activation of the radiation-responsive promoter in the *cre*-encoding vector. The increased cell killing at 5Gy may reflect synergism between radiation and activated GCV.

METHODS and MATERIALS - Summary

For completeness there now follows an outline or summary of some of the methods, techniques and materials which have generally been used in development and testing of this invention unless stated otherwise.

Cells

All bacterial cloning was carried out in *E.coli* strain XL-1 blue MRF' (Stratagene).

The human cell line used for transfection was MCF-7 (breast carcinoma) obtained from the American Tissue Culture Collection (HTB No. 22). The DNA used for PCR amplification of the *egrf*-1 promoter elements was extracted from the human cervical carcinoma line, HeLa.

Plasmids

The pCI and pCI-neo plasmids were obtained from Promega.

The pEGFP-1 plasmid was obtained from Clontech.

The pBS185 (containing the *cre* recombinase gene) and pBS302 (Stop cassette) plasmids were obtained from Gibco BRL™ (Life Technologies, Inc.).

The plasmid clone pBR-*tk* was produced by cloning the nucleotide sequence of the *Herpes simplex* virus type 1 (HSV) thymidine kinase (*tk*) gene, strain CL101, (described by Wagner *et al.*, (1981) as previously mentioned) into Gibco plasmid pBR322, using standard methods.

Enzymes

All restriction endonucleases were obtained fron MBI Fermentas, with the following exceptions. *Sgf*I and I-*Ppo*-I were from Promega. *Acc*I, *Asc*I and *Spe*I were from New England Biolabs.

T4 DNA ligase, Taq DNA polymerase and Klenow were from MBI Fermentas. Expand High Fidelity polymerase was from Boehringer Mannheim. Taq Plus Precision was from Stratagene.

All enzymes were used in the buffers supplied and in accordance with the manufacturers instructions.

DNA Purification

High molecular weight HeLa DNA was extracted from cultured cells using the MBI Fermentas Genomic G2 kit. Large-scale plasmid DNA preparations were carried out using the Qiagen Maxi-purification kit.

DNA extraction and purification from agarose gels (Seakem GTG agarose, FMC Bioproducts) was carried out using the Qiagen™ Gel Extraction kit.

After restriction endonuclease digestion or modification, DNA was routinely purified using the Nucleon PCR/Oligo Clean-up kit.

Nucleotides

Deoxynucleotides for PCR were obtained from Pharmacia and used at 0.2mM final concentration.

Synthetic oligonucleotides for providing the radiation responsive elements and PCR primers less than 60 base pairs in length were obtained from Gibco BRL™. Those of a greater length were obtained from Cruachem.

Transformation of *E.coli*

The procedure used to prepare competent cells for molecular transformation and freezing was that outlined in the previously mentioned reference of Sambrook *et al.* (1989), with a 10mL 0.1M magnesium chloride cell pellet resuspension step prior to the first calcium chloride stage to increase transformation efficiency. The transformation procedure itself was also from this source.

Growth and transfection of human cells

Pre-confluent human tumour cells (MCF-7 [ATCC HTB 22]) grown in RPMI tissue culture medium + 10% foetal calf serum (FCS) were washed with PBS, serum free media (SFM) and then exposed to a transfection mixture of 15μL lipofectamine (Gibco BRL™) mixed with 85μL double-distilled water ($ddH_2O$) combined with 10μL (5μg) of plasmid DNA mixed with 90μL $ddH_2O$. This addition was immediately followed by adding 800μL RPMI + 5% FCS and incubating at room temperature for 5 hours. Subsequently, 1mL of RPMI + 15% FCS was added for 21 hours, after which the medium was removed and replaced with fresh complete RPMI + 10% FCS.

Irradiation

Cells were irradiated at 37°C with 5Gy and 10Gy (2 x 5Gy) of cobalt-60 (gamma-rays) at a dose rate of 1 Gy $min^{-1}$.

FACS analysis

Green Fluorescent Protein (GFP) expression was assessed by fluorescent activated cell sorting (FACS) and scanning (Becton-Dickinson FACScan™: excitation 488 nm). Monolayers of cells were trypsinised, washed with PBS (phosphate buffered saline) and finally resuspended in PBS as a single cell suspension. FITC fluorescence was measured at 530 ± 15 nm, and cell debris resulting in forward and side scatter was gated out. The gated fluorescence 1 profile of the FACScan™ software was used to determine the number of fluorescent cells in the sample.

MTT assay

Cytotoxicity assays were performed using a modification of a method previously published (Morten *et al.*, 1992 "Upregulation of $O^6$-alkyl-guanine-DNA-alkyltransferase expression and the presence of double minute chromosomes in alkylating agent selected Chinese hamster cells" *Carcinogenesis* 13(3): 483-487). Briefly, confluent cultures of cells were trypsinised, twice washed in PBS and resuspended to 2000 cell/mL in complete tissue culture medium (RPMI + 10% foetal calf serum) containing 50 mM ganciclovir (GCV). Aliquots (200 µL) of this suspension were pipetted into each well of 96 well microtitre plates. The plates were incubated at 37°C for 4 days, after which the medium was aspirated and replaced with 100 µL of a 3 mg/mL solution of MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] in PBS for 3 hours, followed by 200 µL of DMSO. The plates were agitated to ensure complete dissolution, and were read on a multiplate reader (Flow Ltd.) at 530 nm and 690 nm.

DNA Manipulations

(a) *PCR:*

The *tk* ORF was obtained by PCR using the specific primers shown in

TABLE 2. The proof-reading DNA polymerases, Expand High-fidelity and Taq Plus PCR were used to avoid introduction of mutations. After 2 minutes of initial DNA strand melting at 94°C, polymerase was added. Amplification was carried out for 15 cycles under the following conditions; 94°C 1 min, 55°C 1 min, 72°C 1min. The latter, elongation, step was extended by 20 seconds each cycle for the last 5 cycles. A single 5 min elongation was also added as a final step. Products amplified from plasmid DNA were agarose gel purified to remove the original template.

(b) *DNA Restriction endonuclease digestion*:

As already described, a series of steps was needed to produce the pComplete1 construct. Briefly, to recap. and summarise:

(i) The Stop cassette was excised from pBS302 with *Eco*RI and *Spe*I and was cloned into the *Eco*RI and *Xba*I sites of pCI-*neo* to produce pStop (see Figure 4).

(ii) The *tk* ORF was amplified from the HSV plasmid clone pBR-*tk*, using the *tk* PCR primer pair shown in TABLE 2. The purified PCR fragment was then treated with *Cla*I and *Not*I and cloned into *Acc*I/*Not*I digested pCI-*neo* to generate pStop-*tk* (also see Figure 4).

*(iii)* The radiation-responsive *egr-1* elements (RR-elements(a)) were cloned into the pCI-neo plasmid as described, using the *Bgl*II and *Cgf*I sites to generate pEGRL(a)-*neo*.

*(iv)* The Cre recombinase ORF was excised from pBS185 with *Xho*I and *Mlu*I and cloned into those sites in pEGRL(a)-*neo* to generate pEGRL(a)-*cre*.

(v) The *Not*I site was removed from the pEGRL(a)-*cre* plasmid by *Not*I digestion. This was followed by "filling-in" of single-stranded overhangs using Klenow, then re-ligation.

(vi) The *Bgl*II/*Bam*HI fragment of the pCI plasmid, containing the CMV I.E. enhancer/promoter, multiple cloning site and SV40 polyadenylation site, was isolated and then cloned into the *Not*I-deletion construct via partial *Bam*HI digestion, allowing insertion downstream of the neomycin resistance gene to generate pEGRL-*cre*MCS2.

(vii) Finally, the *Eco*RI/*Not*I Stop-*tk* cassette of pStop-*tk* was cloned into the inserted multiple cloning site in pEGRL*cre*MCS2 to produce pComplete1.

(c) *Ligation*:

For ligations involving only single-stranded terminal overhangs, 20-50ng of plasmid vector DNA was added to 60-200ng of potential insert DNA and incubated at 21-25°C for approximately 20 hours before transformation. Blunt-end ligations were performed at 4°C.

The radiation responsive element containing oligonucleotides were annealed by placing 0.2-0.5nmoles of each complementary molecule in a 5μL total volume and heating to 55°C for 5min before leaving to cool for about 24hrs. This mixture was then added to 50-100ng of plasmid vector for ligating as normal.

CLINICAL APPLICABILITY

In clinical use the vectors of the present invention will be administered to a cancer patient in a suitable dose and in a suitable pharmaceutical composition using, as already explained, either a virus-based or virus-free method of DNA delivery.

After administration of the composition containing the vector to the patient an appropriate time is allowed for uptake and incorporation into cells. The patient's tumour may then be subjected to a suitable dose of diathermy or ionizing radiation. In the latter case, this is preferably applied using conformal radiotherapy apparatus, or a tumour-targeting radiolabelled agent such as a tumour specific antibody or cytokine may be used. In the case that the tumour has metastasised, whole body diathermy or radiation or a tumour-targeting radiolabelled agent will be used. The heat or radiation activates the heat or radiation responsive promoter with the result that recombinase protein is expressed. Although this expression may be transient, the action of the recombinase results in the recombination of the recombinase target sites and hence excision of the Stop cassette, resulting in the permanent expression of the tumour-sensitizing gene or genes as hereinbefore described in connection with the first embodiment of Figure 1.

When a prodrug activating enzyme is encoded by the sensitizing gene(s), after administration of the vector composition in the course of clinical use and application of heat or ionizing radiation as the case may be, an appropriate therapeutically effective dose of the prodrug is administered in a conventional formulation by any suitable route. This then brings about the death of the cells that express this prodrug activating enzyme, and also cells in the vicinity by virtue of the bystander effect.

In the alternative, if the tumour killing or sensitizing gene encodes proteins such as for example interleukin-2 (IL-2) or granulocyte-macrophage colony-stimulating factor, an immune response will be stimulated in the host that should eradicate the tumour cells. On the other hand, if the tumour killing or sensitizing gene encodes a cytotoxic protein, expression can result directly in the killing of the host tumour cells.

In the case that the tumour sensitizing gene encodes a ribozyme or an antisense RNA molecule, these would be designed to bind to and cleave, or elicit the cleavage of, specific messenger RNA molecules that encode proteins the non-production of which would lead to the death of the cells or render the cells more sensitive to killing by exogenous agents or ionising radiation. However, with this approach it is likely that only those cells to which the therapeutic DNA is delivered would be killed or sensitised, whereas with prodrug activation bystander effects can occur.

The advantages of the invention include the fact that recombination and subsequent activation of the vector only occurs in the region of diathermy or in the irradiated area. The uptake of the vector into normal cells at distant sites will not therefore result in the ability of such cells to activate prodrugs or otherwise elicit tumour sensitisation. Furthermore, very low sub-therapeutic doses of ionizing radiation can be effective in inducing expression of, for example, the *egr-1* enhancer/promoter. Hence, in the case that the tumour has metastasised, tumour-seeking radiolabelled agents that are inefficient in killing tumour cells *per se* can be used to instigate the recombination events and enable the activation of prodrugs, or the other cancer cell-killing processes described, in potentially all metastatic sites as well as in the primary tumour.

As will be seen, the invention presents a number of different aspects and it should be understood that it embraces within its scope all novel and inventive features and aspects herein disclosed, either explicitly or implicitly and either singly or in combination with one another. Also, many detail modifications are possible and, in particular, the scope of the invention is not to be construed as being limited by the illustrative example(s) or by the terms and expressions used herein merely in a descriptive or explanatory sense.

TABLE 1

Synthetic single-stranded oligonucleotides used to produce double-stranded molecules containing radiation responsive elements (RRelements) provided by repeats of the decamer: 5' CCTTATTTGG (SEQ ID NO:1). Plasmid pCompletel contains a total of 6 elements arranged in series in a tandem array:

EGRE 1

*Bgl*II(part of)

5' *GATCT*CCTTA TTTGGCCTTA TTTGGCCTTA TTTGGCCTTA TTTGGCCTTA TTTGGCCTTA TTGG*GCGAT*

*Sgf*I(part of)

(SEQ. ID NO: 2)

EGRE 2 (complementary to EGRE1)

5' *CGC*CCAAATA AGGCCAAATA AGGCCAAATA AGGCCAAATA AGGCCAAATA AGGCCAAATA AGG*A*

(SEQ. ID NO:3)

Examples of similar oligonucleotides containing four repeats of SEQ. ID NO: 1

EGRE 3

*Bgl*II (part of)

5' *GATCT*TTATT TGGCCTTATT TGGCCTTATT TGGCCTTATT TGG*GCGAT*

*Sgf*I(part of)

(SEQ. ID NO: 4)

EGRE 4 (complementary to EGRE 5)

5' *CGC*CCAAATA AGGCCAAATA AGGCCAAATA AGGCCAAATA AGGCCAAATA AGG*A*

(SEQ. ID NO: 5)

TABLE 2

A. Primers used for PCR amplification of promoter/enhancer sequences of human primary response gene *egr-1*/TIS8.

For enhancer/promoter:

EGRE 5

*Bgl*II

5' TCC*AGATC*TC CCGGTTCGCT CTCACGGTCC CTGAGG 3'

(SEQ. ID NO: 6)

EGRP 6

*Asc*I

5' C*GGCGCGCC*G CTGGATCTCT CGCGACTCCC CG

(SEQ. ID NO: 7)

For enhancer alone:

EGRE 7

*Sgf*I

5' A*CTGCGATCG C*GGGCCCGGC CCGGCCCGCA TCCCAGGCCC CC

(SEQ. ID NO: 8)

B. Primers used for PCR amplification of Thymidine kinase gene

Cla*tk*:

*Cla*I

5' CC*ATCGAT*AT GGCTTCGTAC CCCGGC

(SEQ. ID NO: 9)

*tk*Not:

*Not*I

5' AAGGAAAAAA *GCGGCCGC*CT CCTTCCGTGT TTCAGTTAGC

(SEQ. ID NO: 10)

REFERENCES

For convenience, some of the references mentioned in the foregoing description are listed below:-

Morten-JE; Bayley-L; Watson-AJ; Ward-TH; Potter-PM; Rafferty-JA; Margison-GP (1992), "Upregulation of $O^6$-alkylguanine-DNA-alkyltransferase expression and the presence of double minute chromosomes in alkylating agent selected Chinese hamster cells". *Carcinogenesis*. 13(3): 483-7

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning - A Laboratory Manual. Cold Spring Harbor Laboratory Press, U.S.A.*

Datta, R., Rubin, E., Sukhatme, V., Quershi, S., Hallahan, D. E., Weichselbaum, R. R. & Kufe, D. (1992), *Proc. Natl. Acad. Sci. USA* 89, 101149-101153

Elizabeth A. Austin, *et al.*, (1992), "A First Step in the Development of Gene Therapy for Colorectal Carcinoma: Cloning, Sequencing, and Expression of *Escherichia coli* Cytosine Deaminase", *Molecular Pharmacology*, 43, 380-387)

D.E. Hallahan *et al.* (1995) "Spatial and temporal control of gene therapy using ionizing radiation", *Nature Medicine*, Vol. 1, No. 8, 786-791) and D. E. Hallahan, *et al.*, (1995), "*c-jun* and *Egr-1* Participate in DNA Synthesis and Cell Survival in Response to Ionizing Radiation Exposure", *The Journal of Biological Chemistry*, 270, 30303-30309)

Martina Anton, *et al.*, (1995), "Site-Specific Recombination Mediated by an Adenovirus Vector Expressing the Cre Recombinase Protein: a Molecular Switch for Control of Gene Expression", *Journal of Virology*, 69, 4600-4606, and Minmin Qin, *et al.*, (1995), "Site-specific cleavage of chromosomes in vitro through Cre-Lox recombination", *Nucleic Acids Research*, 23, 1923-1927

Dymecki (1996) "Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice", *Proc. Natl. Acad. Sci. U.S.A.*, 93, 6191-6196

E. R. Schuur, *et al.*, (1996), "Prostate-specific Antigen Expression Is Regulated by an Upstream Enhancer", *The Journal of Biological Chemistry*, 271, 7043-7051

Tsutsumi-Ishii *et al.*, (1995) *Cell growth and differentiation*, 6, 1-8) and the MDR-1 promoter (Zastawy, R.L. (1993) *Oncogene* 8, 1529-1535

M.J. Parr *et al.*, (1997) (see "Tumour-selective transgene expression in vivo mediated by an E2F-responsive adenoviral vector", *Nature Medicine*, 3, 1145-1149

S. M. Bailey *et al.*, (1996) "Investigation of alternative prodrugs for use with *E. coli* nitroreductase in 'suicide gene' approaches to cancer therapy" *Gene Therapy*, 12, 1143/1150

Friedman in *Therapy for Genetic Disease*, T. Friedman, ed., Oxford University Press (1991) and reviewed by I.M.Verma and N. Somia (1997) in "Gene therapy-promises, problems and prospects" *Nature* 389, 239-242

Weichselbaum *et al.* (1994)) "Gene therapy targetted by radiation preferentially radiosensitizes tumour cells", *Cancer Research,* 54, 4266-4269

Wagner, M.J. *et al.*, (1981) "Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type-1", *Proc. Natl. Acad. Sci. USA*, 78, 1441-1445

Sakamoto *et al.*, "5' Upstream sequence and genomic structure of the human primary response gene *egr*-1/TIS8" (1991) *Oncogene* 6, 867-871

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED
(B) STREET: CAMBRIDGE HOUSE, 6-10 CAMBRIDGE TERRACE, REGENT'S PARK,
(C) CITY: LONDON
(E) COUNTRY: UNITED KINGDOM
(F) POSTAL CODE (ZIP): NW1 4JL

(ii) TITLE OF INVENTION:
IONIZING RADIATION OR DIATHERMY-SWITCHED GENE THERAPY VECTORS AND THEIR USE IN ANTITUMOUR THERAPY

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCTTATTTGG 10

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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TTGGGCGAT 69

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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AGGA 64

(2) INFORMATION FOR SEQ ID NO: 4:

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(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

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(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CGGCGCGCCG CTGGATCTCT CGCGACTCCC CG 32

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ACTGCGATCG CGGGCCCGGC CCGGCCCGCA TCCCAGGCCC CC 42

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

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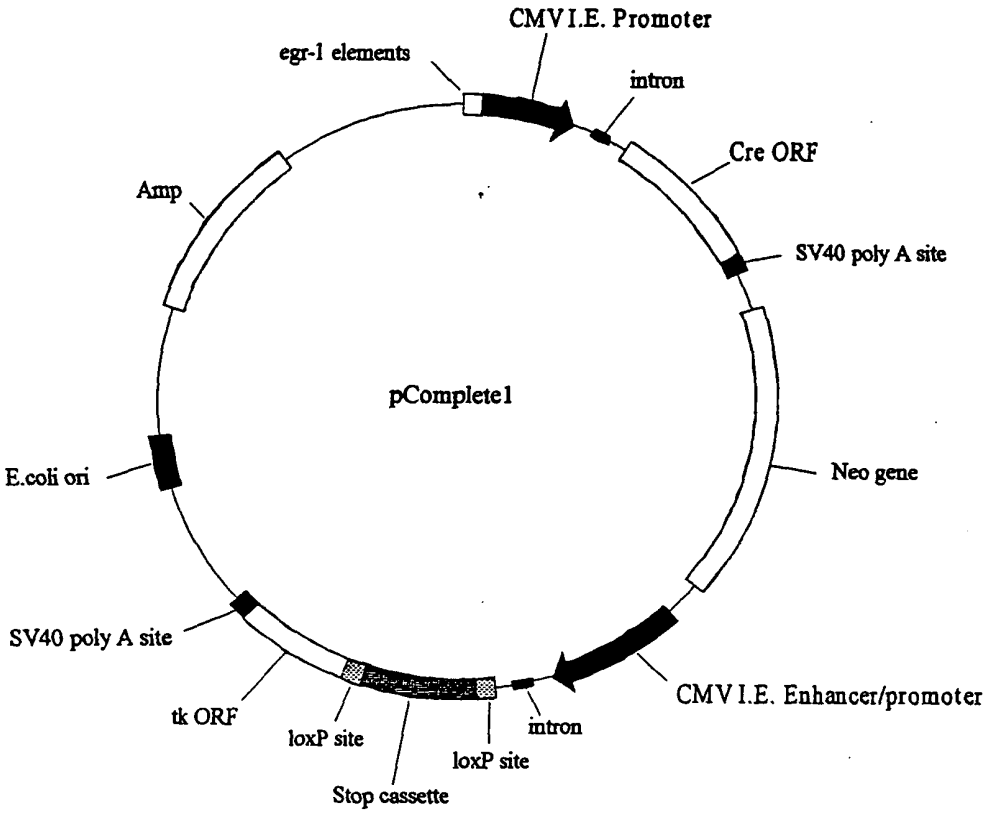


Figure 1. Structure of the plasmid pComplete1. Not to scale

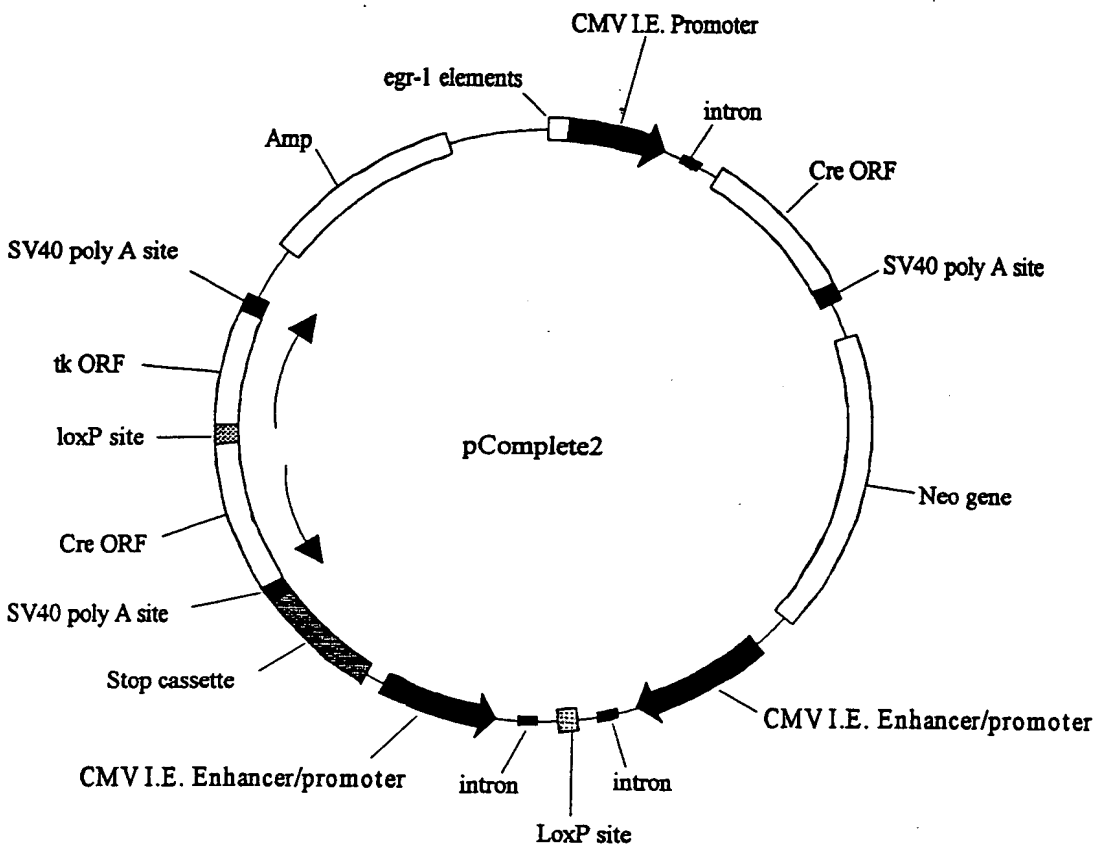


Figure 2. Structure of the plasmid pComplete2. The curved arrows adjacent to the Cre and tk ORFs indicate the direction of Cre and tk transcription following recombination at the loxP sites. Not to scale.

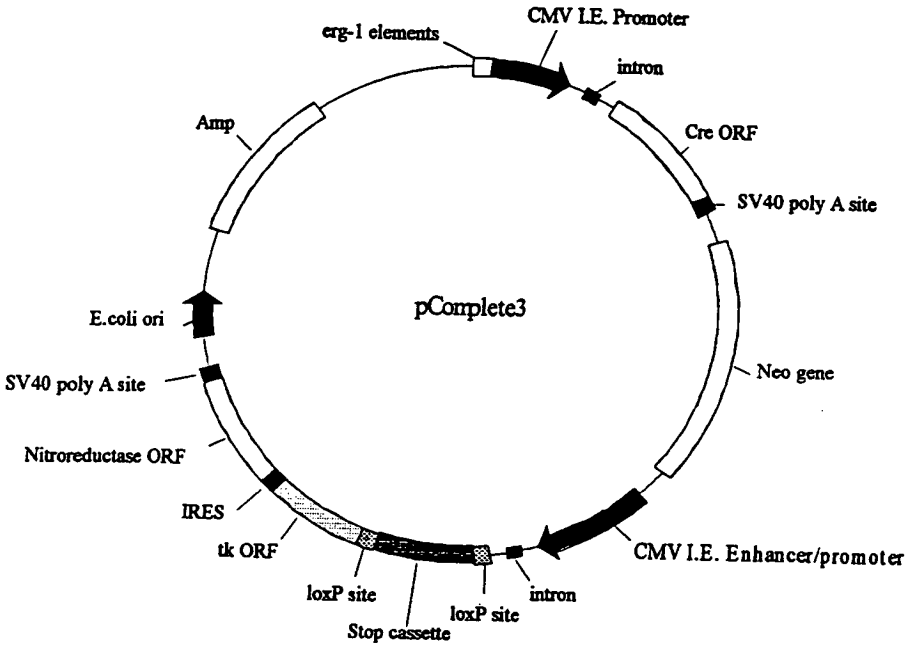


Figure 3. Structure of the plasmid pComplete3. Not to scale

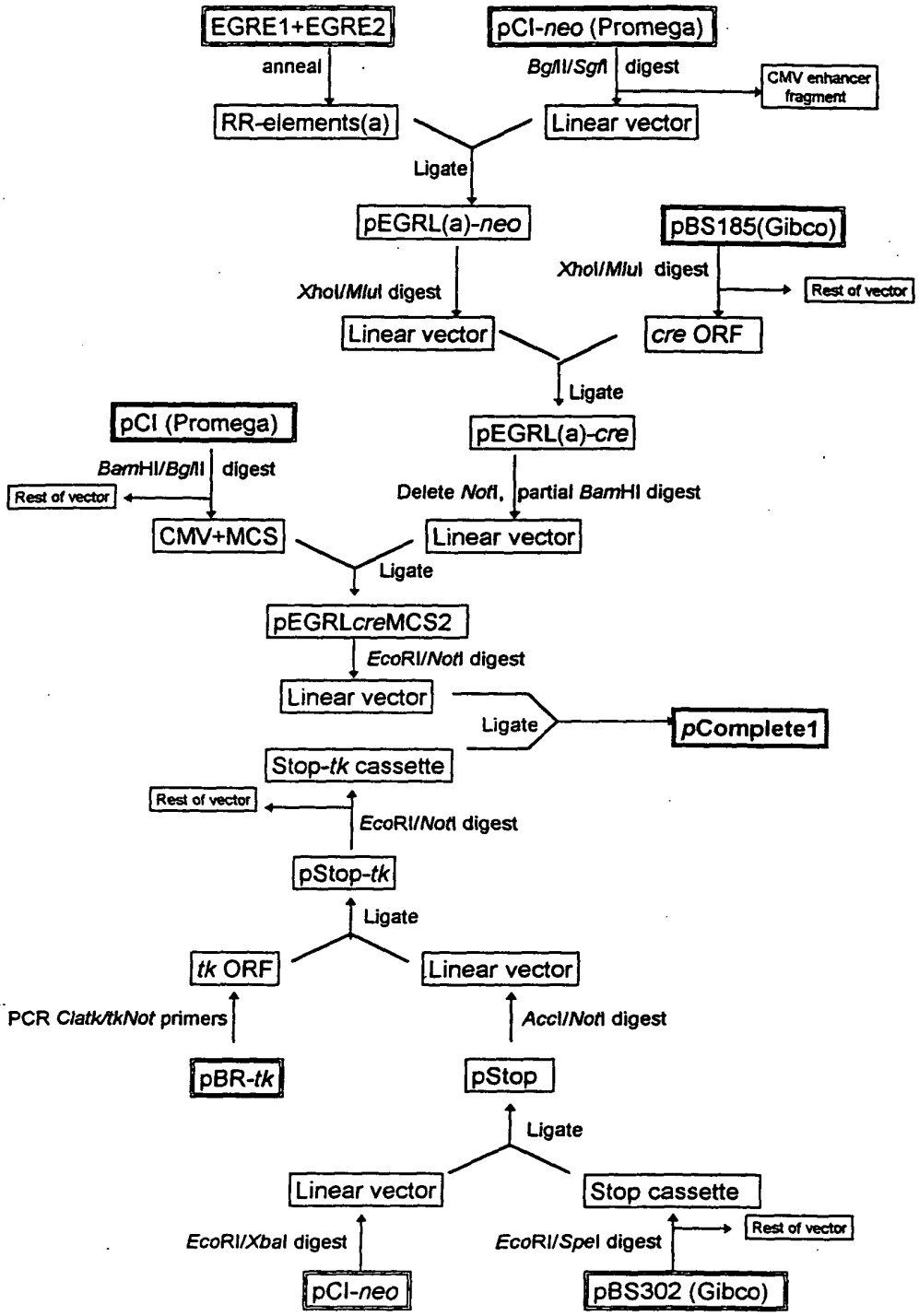


Figure 4. Scheme for the construction of a version of pComplete containing tandemly arranged radiation-responsive elements of the *egr-1* promoter. Double-framed boxes indicate starting materials.

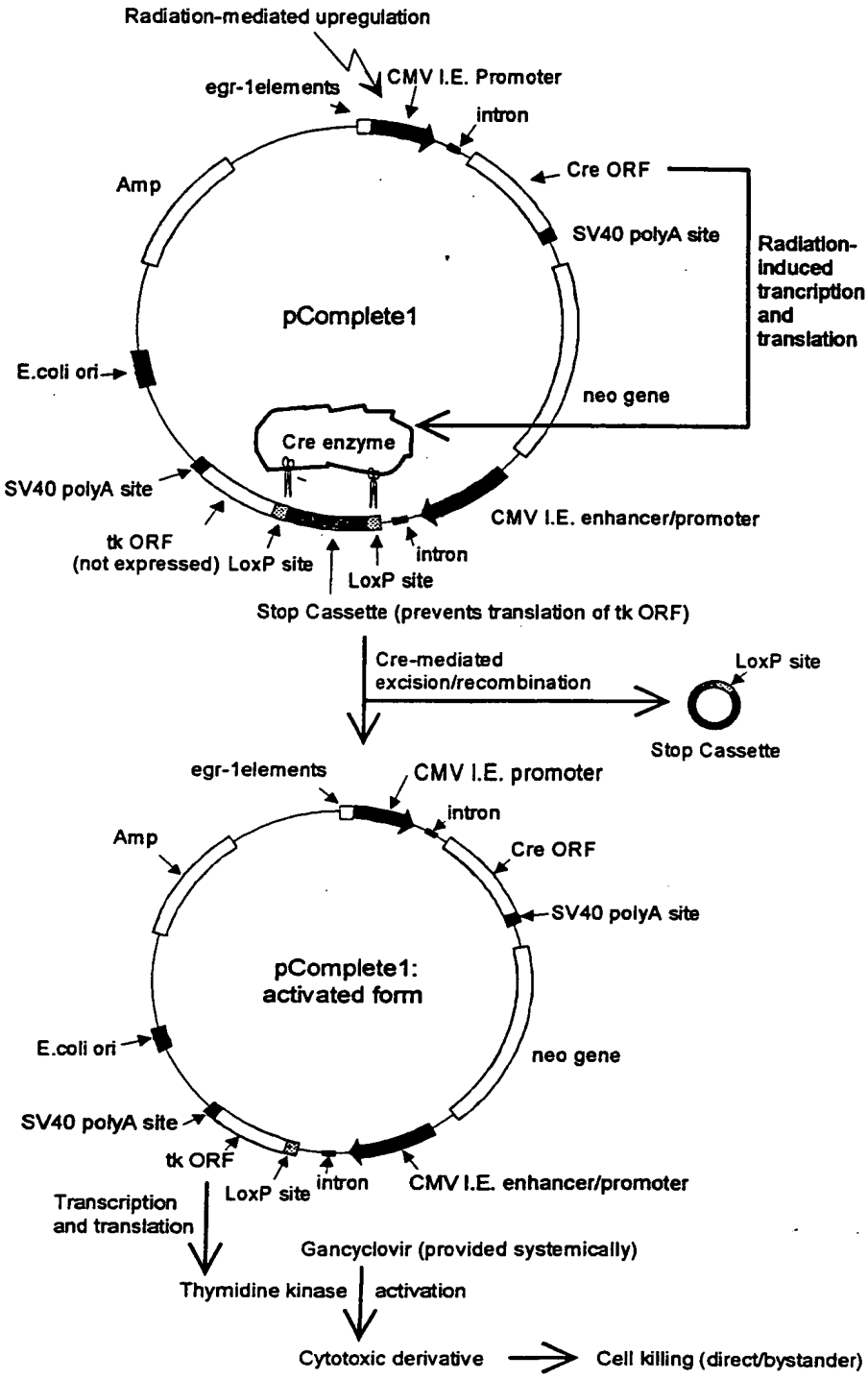


Figure 5. Mechanism of recombination-mediated activation of pComplete1 with consequent thymidine kinase expression, gancyclovir activation and cell killing.

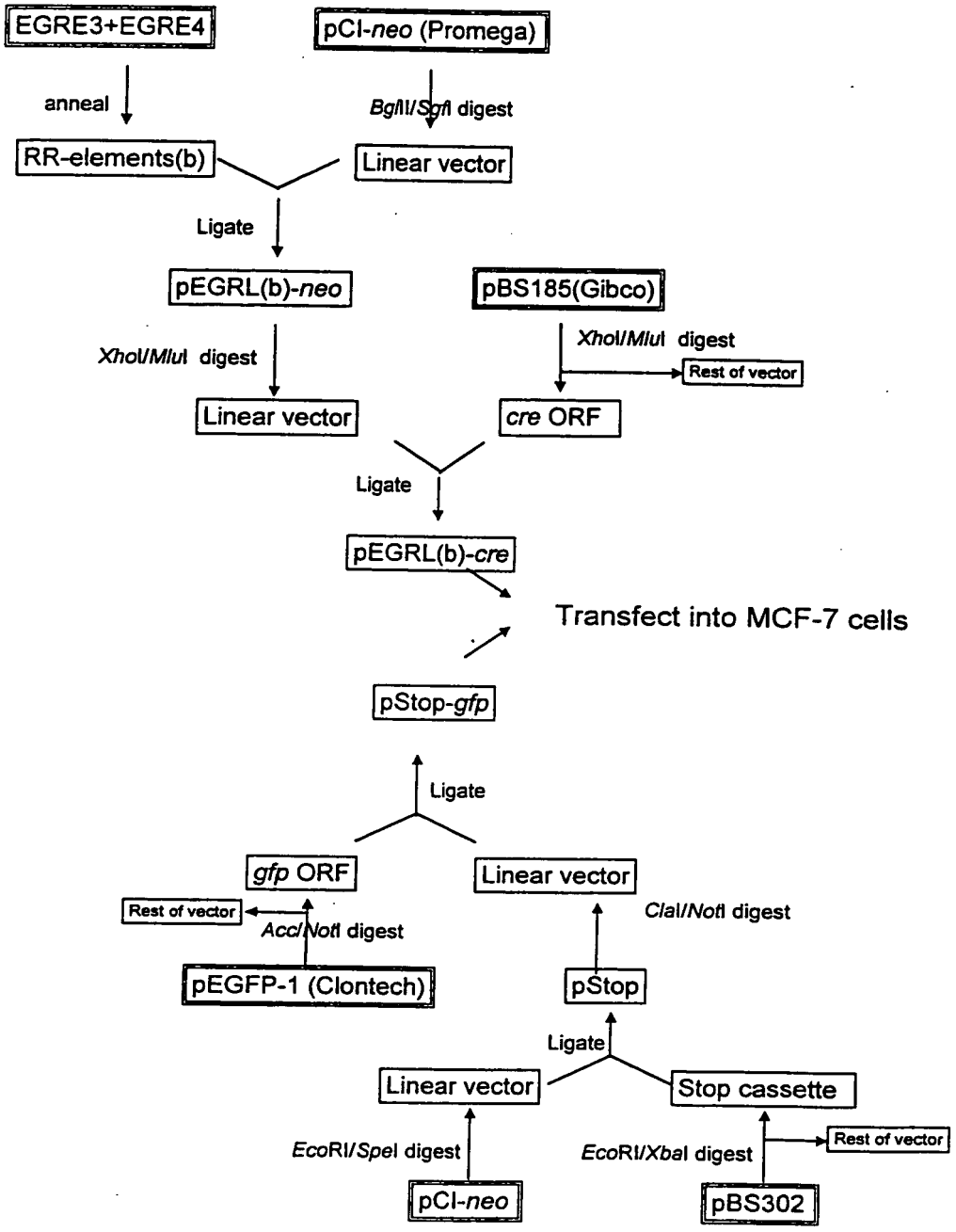


Figure 6. Scheme for the construction of pStop-*gfp* and pEGRL(b)-*cre* which were introduced into MCF-7 cells to generate the data shown in Figure 7. Double-framed boxes indicate starting materials.

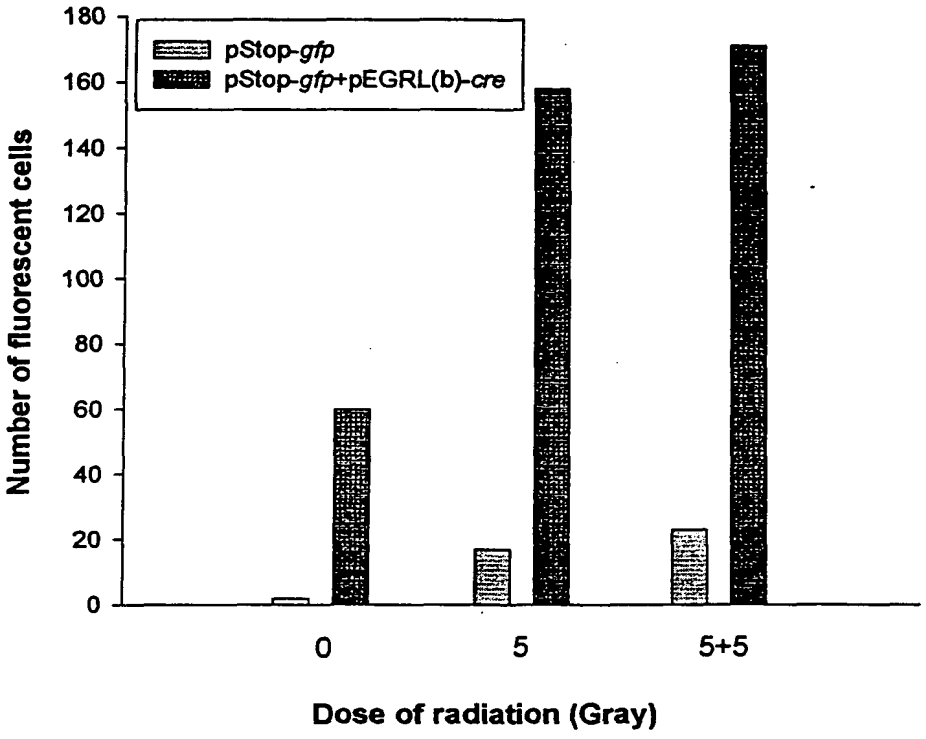


Figure 7. Number of fluorescent pStop-*gfp*-transfected (light grey bars) and pStop-*gfp* plus pEGRL(b)-*cre*-transfected (dark grey bars) MCF-7 cells irradiated at the doses indicated.

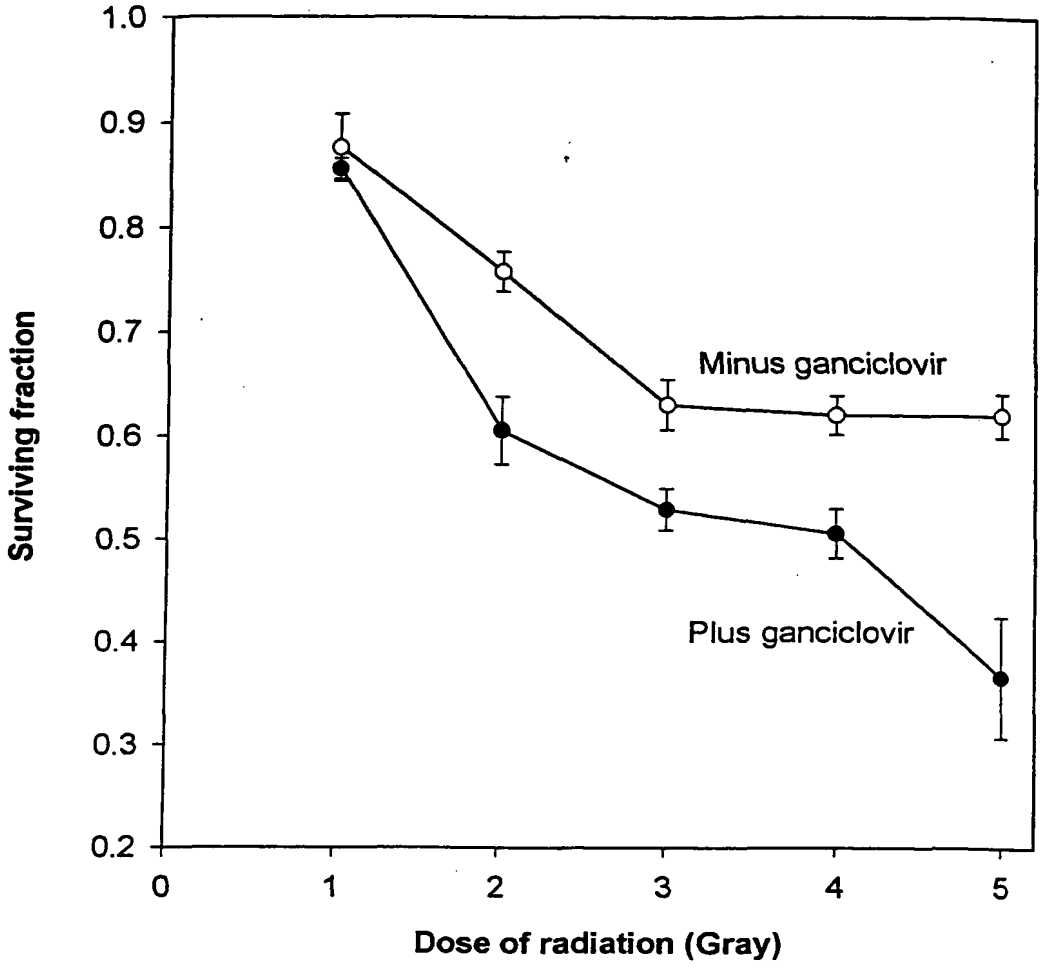


Figure 8. Survival of pStop-*tk*-transfected pCE cells (*i.e.* harbouring pEGRL(b)-*cre*) irradiated at the doses indicated in the presence (closed circles) or absence (open circles) of ganciclovir. Error bars indicate standard deviation.

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